Sulfohydrolase Activity and Carrageenan Biosynthesis in *Chondrus crispus* (Rhodophyceae)[1]

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ABSTRACT

An enzyme catalyzing the conversion of μ- to κ-carrageenan has been demonstrated in both haploid and diploid plants of *Chondrus crispus*. It acts at the polymer level producing 3,6-anhydro-D-galactose with the stoichiometric release of sulfate. Two-thirds of the recoverable enzyme was associated with the 15,000g pellet most of which could be solubilized by passage through a Ribi Cell Fractionator. The enzyme precipitated between 2.65 and 4.24 M $(NH_4)_2SO_4$ and was partly purified on DEAE-cellulose columns. This sulfohydrolase has a pH optimum near 6.5 and is inhibited by molybdate, phosphate, sulfate, tungstate, cysteine, ATP, GTP, UDP, and by λ-carrageenan. No activator was found. The enzyme showed a similar affinity for several preparations of μ-carrageenan and for the κ-carrageenase-resistant fraction from κ-carrageenan thus confirming that the latter is a biosynthetically unfinished molecule.

A comparable extract from *Gigartina stellata* gave a higher specific activity for the sulfohydrolase, but was otherwise quite similar to the *Chondrus* enzyme.

Cell walls of red seaweeds frequently contain conspicuous amounts of galactans among which the carrageenans are a commercially important group. These polysaccharides are composed of regularly alternating α-1,3; β-1,4-linked D-galactose residues which differ in degree and position of sulfate esterification, and in their content of 3,6-anhydrogalactose (12). As early as 1963 Rees (11) postulated that a "λ-like" fraction from *Chondrus crispus* may be the biological precursor of κ-carrageenan in a manner analogous to that seen in the biosynthesis of porphyran. In the latter case L-galactose-6-sulfate residues in the polymer were converted to 3,6-anhydrogalactose by an enzyme present in *Porphyra umbilicalis* (10). The predicted enzyme activity was detected in extracts of *Gigartina stellata* (6), but similar preparations from *C. crispus* appeared to be inactive (Rees, personal communication).

The present paper is the first characterization of an enzyme from *C. crispus* which eliminates sulfate from μ-carrageenan to form the 3,6-anhydrogalactose of κ-carrageenan. The enzyme was not sufficiently characterized to be listed in the 1972 Enzyme Nomenclature. Because the mechanism of the reaction is unknown the name "sulfohydrolase" used in the present report is based on the release of sulfate from the polymer.

MATERIALS AND METHODS

Plant Materials. *C. crispus* Stackhouse, strain NRC-T4, was cultured in tanks essentially as described by Neish and Shacklock (9). Wild haploid plants of *C. crispus* and *Gigartina stellata* (Stackhouse) Batters were collected in May at Morris Point, Halifax County, Nova Scotia while diploid plants of *C. crispus* were collected in the same month at Toney River, Pictou County, Nova Scotia. Carrageenans were isolated and purified according to methods of Craigie and Leigh (3).

Enzyme Extraction from Haploid Plants. Apical 2- to 3-cm pieces of *C. crispus* (100 g) were frozen in liquid N_2 and pulverized in a ball mill for 2 min. The resulting flour was allowed to thaw and 150 ml of cold buffer containing 0.05 M Tris-HCl, 0.5 M KCl, and 0.01 M 2-mercaptoethanol (pH 9.5) were added. The suspension was stirred for 2 hr and left overnight in crushed ice. The crude homogenate was recovered after centrifugation at 15,000g for 20 min. Cold saturated $(NH_4)_2SO_4$ was added and the fraction precipitating between 2.65 and 4.24 M was collected by centrifugation. The precipitate, containing some polysaccharides as well as the active protein, was dissolved in 25 ml of 0.05 M Tris-HCl with 2-mercaptoethanol (pH 7.1) and dialyzed overnight in the same buffer. This enzyme preparation was routinely used.

Purification of DEAE-Cellulose. The protein (150 mg) from the $(NH_4)_2SO_4$ precipitation was applied to a column (25 × 3 cm) of DEAE-cellulose previously equilibrated with 0.05 M borate containing 0.01 M 2-mercaptoethanol (pH 7.5). The column was washed with 250 ml of the same buffer followed with 1 M KCl. Three-ml fractions were collected every 1.6 min. Fractions 141 to 159 containing the active enzyme were pooled and treated with saturated $(NH_4)SO_4$ until 4.24 M was reached. The precipitate collected by centrifugation was redissolved in 0.05 M Tris-HCl containing 0.01 M 2-mercaptoethanol (pH 7.1) and dialyzed overnight in the same buffer.

Preparation of Soluble and Particulate Fractions. The supernatant, S_1, from centrifugation of the crude extract at 15,000g, was centrifuged at 100,000g in a Spinco model L ultracentrifuge. The clear purplish supernatant (S_2) was dialyzed overnight in 0.05 M Tris-HCl with 0.01 M 2-mercaptoethanol (pH 7.1). The particulate fraction was resuspended in 0.05 M Tris, 0.005 M EDTA and 0.01 M 2-mercaptoethanol (pH 9). After dialysis overnight in Tris-HCl-mercaptoethanol buffer (pH 7.1), this suspension (P_1) was clarified by centrifugation at 15,000g before use.

The residue from the initial 15,000g centrifugation was suspended in 100 ml of 0.05 M Tris-HCl, 0.5 M KCl, 0.01 M 2-mercaptoethanol (pH 9.5) and passed through a Sorvall Ribi Cell Fractionator model RF-1 at a pressure of 25,000 p.s.i. Another 50 ml of the suspending buffer was added to the homogenate and the mixture was clarified by centrifugation. One subsample of this homogenate was dialyzed overnight in Tris-mercaptoethanol to give fraction RS_1, while another was centrifuged at 100,000g for 2 hr. The supernatant was dialyzed overnight giving fraction RS_2. One portion of the particulate fraction was resuspended in pH 9 buffer as above; a second was washed with acetone (−15 C), dried, and resuspended in the same pH 9 buffer. Both particulate fractions were dialyzed in the pH 7.1 buffer and centrifuged before use. These are fractions RP_1 and RP_2, respectively.

Preparation of Enzyme from Other Sources. The sulfohydrolase was isolated from tetrasporophytes of *C. crispus* by essentially the same method as for the haploid plants except that the ratio of fresh wt to buffer was 50 g in 300 ml of buffer. The dialyzed crude

extract was further purified by stirring it into the cellulose at the top of a DEAE-cellulose column before elution with borate buffer and KCl as outlined for the enzyme from the haploid plants.

The sulfohydrolase from G. stellata was isolated by the procedure described for haploid C. crispus.

Enzyme Assay. Sulfohydrolase activity was assayed by measuring the amount of 3,6-anhydrogalactose produced. Much of our earlier work was done with PIPES[2] buffer, however, it was later found that Tris-maleate buffer gave higher activity and all subsequent assays were done with this buffer. A typical reaction mixture contained 120 mM Tris, 120 mM maleate, 7 mM 2-mercaptoethanol, 0.8 mg of μ-carrageenan and 2.5 mg of extract protein in a total volume of 1 ml. The final pH was approximately 6.5. At intervals, 0.1-ml samples of reaction mixture were killed in 1 ml of 6 N HCl. Samples taken at zero time were used as controls. The increase in 3,6-anhydrogalactose content was determined by the resorcinol procedure (17).

In some experiments the enzyme activity was determined by measuring the sulfate released. An aliquot of reaction mixture (1.5 ml) was centrifuged at 1,000g in a CF 50 A Centriflo membrane cone system (Amicon) for 1 hr followed by a 0.5-ml water wash. The filtrate was assayed for sulfate by a modification of the method of Jones and Letham (4). To a 1-ml sample a microdrop of cetyltrimethyl-ammonium bromide was added, followed by 0.5 ml of 4-amino-4'-chlorobiphenyl · HCl (95 mg in 500 ml of 0.1 N HCl). After 2 hr at room temperature the suspension was shaken and the A was read at 500 nm and referred to a standard curve to determine the amount of sulfate liberated.

Preparation of κ-Carrageenase. A culture of Pseudomonas carrageenovora was kindly provided by W. Yaphe, McGill University, Montreal. κ-Carrageenase was prepared to the first DEAE-cellulose step as described by Weigl and Yaphe (16).

Preparation of the Enzyme Resistant Fraction. A 2-g sample of κ-carrageenan in 250 ml of 0.05 M NaH$_2$PO$_4$ (pH 7.6) was incubated with the purified κ-carrageenase at 37 C. The reaction mixture was tested every 2 hr for increases in reducing power measured as galactose by the Nelson-Somogyi procedure (14). Purified κ-carrageenase was added at intervals until there was no further increase in reducing power. The digest was clarified by centrifugation. Sodium acetate was added to a concentration of 0.1 M and the enzyme resistant fraction was precipitated by the slow addition of 3 volumes of 2-propanol. After settling overnight most of the supernatant liquid was siphoned off and the precipitate was collected by centrifugation. This was redissolved in water and dialyzed 48 hr against several changes of distilled H$_2$O. The lyophilized material (0.98 g) is the enzyme resistant fraction (16).

Measurement of Protein. Protein was measured by the biuret or Folin-Ciocalteu methods (7).

RESULTS

Enzyme Fractionation. The sulfohydrolase activity precipitated between 2.65 and 4.24 M (NH$_4$)$_2$SO$_4$ with only traces of activity occasionally found between 0 and 2.65 M. The (NH$_4$)$_2$SO$_4$ precipitate did not show an absolute requirement for μ-carrageenan since 80% of the activity was detected without the addition of the polysaccharide substrate. The enzyme eluted from the cellulose column with KCl showed an absolute requirement for the substrate (Table I). It was not active with λ-carrageenan, but did react with the ERF obtained by κ-carrageenase treatment of κ-carrageenan. No activity was detected after boiling the enzyme for 15 min.

Enzyme Activity. The specific activity of the enzyme was low, consequently reactions were run for several hr. Under the experimental conditions described, the activity is linear for at least 4 hr.

[2] Abbreviations: ERF: enzyme-resistant fraction as described under "Materials and Methods"; PIPES: piperazine-N-N'-bis(2-ethanesulfonic acid).

No activity was detected if the crude extract was not dialyzed. A 3- to 5-fold increase in specific activity was obtained when the residue, after overnight steeping in buffer, was passed through the Ribi Cell Fractionator. Presumably this treatment increased the breakage of membranes and organelles thus releasing more enzyme into solution.

Stability. The sulfohydrolase dissolved in 0.05 M Tris with 0.01 M 2-mercaptoethanol (pH 7.5) lost 50% of its activity after 6 days at 4 C. If stored in the same buffer at pH 7.1 and −15 C, the enzyme lost 10% of its activity in 1 week and 20% after 18 days. When suspended in saturated (NH$_4$)$_2$SO$_4$ at −15 C there was very little change in specific activity after 3 weeks.

Effects of pH and Temperature. The effects of pH using various buffers are shown in Figure 1. Maximum activity occurred near pH 6.5 and this pH was used in all our assays.

The sulfohydrolase was assayed at 22, 30, 40, and 60 C with maximum activity occurring at 40 C and none at 60 C.

Effect of Various Compounds. Numerous compounds were examined as potential activators or inhibitors. Table II shows the

Table I. Purification of a Sulfohydrolase from Haploid Chondrus crispus

Reaction was carried out for 4 hr at 30 C with or without added μ-carrageenan and with enzyme resistant fraction (ERF) or λ-carrageenan replacing the μ-carrageenan.

Enzyme	Substrate	Specific Activity µg mg⁻¹ Protein	Total Activity µg	% Recovery
Crude extract	(±µ)	1.4	2402	100
(NH₄)₂SO₄ precipitate	(-µ)	7.4		-
(NH₄)₂SO₄ precipitate	(+µ)	9.2	2397	100
DEAE-cellulose purified	(-µ)	0		-
" " "	(+µ)	7.9	420	17.5
" " "	(+ ERF)	10.2		-
" " "	(+ modified ERF)	0		-
" " "	(+λ)	0		-

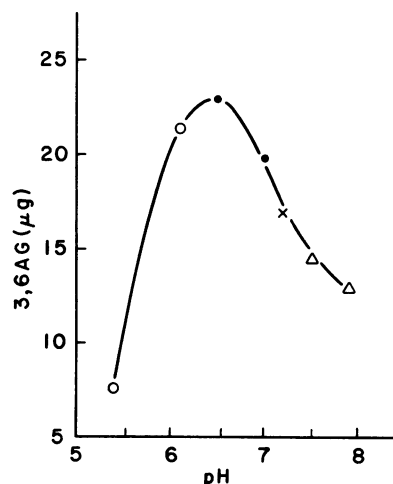

FIG. 1. Effect of pH on sulfohydrolase activity. Buffers: O, MES; ●, PIPES; ×, HEPES; △, Tris. Reaction was measured after 3 hr at 30 C with the (NH$_4$)$_2$SO$_4$-purified enzyme.

Table II. Inhibition of Sulfohydrolase Activity

Reaction mixture contained 0.8 mg μ-carrageenan, 120 mM PIPES buffer (pH 6.5), the inhibitor, and 3.4 mg (NH₄)₂SO₄ purified enzyme in a total volume of 1.0 ml. Incubation was for 3 hr at 30C.

Compound Tested	% Inhibition
K₂HPO₄ (5 mM)	36
Na₄P₂O₇ (5 mM)	28
K₂SO₄ (5 mM)	34
Na₂WO₄ (9 mM)	63
Na₂MoO₄ (9 mM)	32
L-cysteine (8 mM)	36
λ-Carrageenan (0.1%)	44
ATP (5 mM)	100
UTP (5 mM)	50
GTP (5 mM)	60

inhibitory effect of some of the compounds tested. Others such as NaCl, KCl, NH_4Cl, $BaCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, zinc acetate, sodium tetraborate, ADP, AMP, and GMP at a concentration up to 10 mM were without effect. No activators were found.

Stoichiometry of Reactions. The KCl-soluble μ-carrageenan is sulfated at C-4 of the 3-linked D-galactopyranose and C-6 of the 4-linked D-galactopyranose in the repeating disaccharide unit (12). The increase in 3,6-anhydrogalactose observed in the enzyme reaction must be accompanied by the elimination of the sulfate from C-6 of the 4-linked unit. The data of Table III show the liberation of 1 mol of sulfate for each anhydrogalactose produced in the sulfohydrolase reaction.

Effect of Substrate. The effect of varying the concentrations of KCl-soluble carrageenans from haploid *C. crispus* or the ERF from κ-carrageenase digestion of κ-carrageenan is shown in Figure 2. The sulfohydrolase showed a similar affinity for each of the polysaccharide fractions tested. The enzyme was most active with the κ-carrageenase-resistant fraction.

Sulfohydrolase in Soluble and Particulate Fractions. Fractionation by centrifugation at 15,000g showed that 65% of the recoverable activity remained bound in the cell debris after steeping overnight (Table IV). Further disruption of the debris with a Ribi Cell Fractionator solubilized about 84% of the bound enzyme with a 3-fold increase in specific activity. Acetone treatment of the final particulate fraction did not improve recovery of the enzyme.

Distribution of the Sulfohydrolase. The presence of the enzyme was observed in both haploid and diploid *C. crispus* collected in nature. The specific activity of the extract from the wild plants was approximately 30% of that from the cultivated alga.

Table III. Stoichiometry of the Sulfohydrolase Reaction

Reaction mixture for the crude extract contained 73 mM tris, 73 mM maleate, 0.2 mM 2-mercaptoethanol, 0.036% μ-carrageenan and 24.8 mg of protein extract in a total volume of 5.5 ml. Samples were assayed at 0 time and at 24 hr. The partially purified enzyme reaction mixture contained 58.5 mM tris, 58.5 mM maleate, 0.15 mM 2-mercaptoethanol, 0.049% μ-carrageenan and 15.9 mg protein in a total volume of 4.1 ml. Samples were assayed at 0 time and after 7 hr.

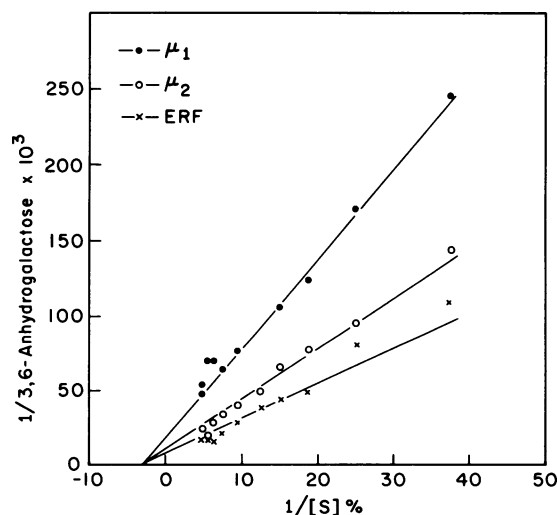| Enzyme	3,6-Anhydrogalactose (μmol)	SO_4^{2-} (μmol)
Crude extract	(1) 0.84	0.81
	(2) 0.71	0.69
$(NH_4)_2SO_4$ purified	(1) 0.67	0.59
	(2) 0.80	0.77

FIG. 2. DEAE-cellulose-purified sulfohydrolase activity with varying concentrations of μ-carrageenan preparations or ERF. Preparation μ_1 was extracted initially from *C. crispus* with 0.5 M $NaHCO_3$ whereas μ_2 was from the same source but extracted with 0.4 M sodium acetate-0.1 M Tris at pH 8.2. Activity was assayed at 30 C after 6 hr.

Table IV. Sulfohydrolase Activity of the Soluble and the Particulate Fractions of Haploid <u>Chondrus</u> <u>crispus</u>.

Details of fractionation are given in Materials and Methods. Enzyme activity was assayed at 30 C as increase in 3,6-anhydrogalactose after 6 hr with μ-carrageenan as the substrate.

Enzyme Fraction	Total Protein μg	Total Activity μg	Specific Activity μg mg^{-1} Protein
S_1	1083	1819	1.68
S_2	782	1009	1.29
P_1	128	202	1.58
RS_1	720	3433	4.77
RS_2	510	2636	5.17
RP_1	97.5	504	5.16
RP_2	33.8	303	8.97

S_1 = 15,000 x g supernate; S_2 = 100,000 x g supernate of S_1; P_1 = 100,000 x g pellet of S_1; RS_1 = 15,000 x g supernate of extracted residue after Ribi Cell fractionation; RS_2 = 100,000 x g supernate of RS_1; RP_1 = 100,000 x g pellet of RS_1; RP_2 = acetone washed 100,000 x g pellet of RS_1.

Table V. Sulfohydrolase Activity from Diploid <u>Chondrus</u> <u>crispus</u>

Enzyme	Substrate	Specific Activity μg mg^{-1} Protein
Crude extract before DEAE chromatography	μ-Carrageenan	Trace
Crude extract before DEAE chromatography	ERF	6.68
Crude extract after DEAE chromatography	μ-Carrageenan	6.02
Crude extract after DEAE chromatography	ERF	12.29
$(NH_4)_2SO_4$ precipitate	μ-Carrageenan	Trace
$(NH_4)_2SO_4$ precipitate	ERF	9.50

Table VI. Sulfohydrolase Activity in <u>Gigartina</u> <u>stellata</u>

Crude extract (0.97 mg protein) was assayed with 80 mM tris, 80 mM maleate, 7 mM 2-mercaptoethanol and 0.8 mg μ-carrageenan in a total of 1.5 ml. An 0.2 ml aliquot of the mixture was used at time 0 and 4 hr for the determination of 3,6-anhydrogalactose. The $(NH_4)_2SO_4$ precipitated enzyme (5.1 mg protein) was assayed with 120 mM tris, 120 mM maleate, 7 mM 2-mercaptoethanol and 0.8 mg of μ-carrageenan in a total of 1.5 ml. The inhibitory effect of λ-carrageenan was tested at 0.05%. A 0.05 ml aliquot of reaction mixture was assayed for 3,6-anhydrogalactose at 0 time and after 4 hr.

Enzyme Fraction	Specific Activity μg 3,6-Anhydrogalactose mg^{-1} Protein
Crude extract (-μ)	Trace
Crude extract (+μ)	13.78
$(NH_4)_2SO_4$ precipitate (-μ)	40.56
$(NH_4)_2SO_4$ precipitate (+μ)	39.30
$(NH_4)_2SO_4$ precipitate (+μ + λ)	12.97

Extracts from the diploid plants were more viscous and thus more difficult to handle than those from the haploid plants. The active protein, however, was similar to that of haploid plants and precipitated between 2.65 and 4.24 M $(NH_4)_2SO_4$.

Using 1 M KCl-soluble μ-carrageenan as the substrate only a trace of activity was detected in the crude extract and in the $(NH_4)_2SO_4$-precipitated fraction. Much more activity was detected in these extracts when the κ-carrageenase-resistant fraction from κ-carrageenan was the substrate. Purification of the crude extract by DEAE-cellulose chromatography resulted in a much more active enzyme as shown in Table V.

Sulfohydrolase activity was also detected in the extract of *G. stellata*. The enzyme activity was precipitated between 2.65 and 4.24 M $(NH_4)_2SO_4$ and was inhibited by λ-carrageenan. The specific activity was higher than the *C. crispus* enzyme; the $(NH_4)_2SO_4$ precipitate was less viscous and did not show a requirement for the substrate (Table VI). The polysaccharide associated with this fraction had more 3,6-anhydrogalactose/unit of protein than the corresponding preparation from *C. crispus*.

DISCUSSION

Several problems peculiar to extracts of red seaweeds were encountered in preparing the enzyme fractions. The cartilaginous

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algal thallus defied the grinding procedures normally used with plant tissues so we placed the alga in liquid N_2 before ball milling the frozen tissue. Copious amounts of anionic polysaccharides were liberated into the buffer and these were difficult to handle owing to their high viscosity and their affinity for protein binding. This problem was partially overcome in haploid plants by using KCl in the isolation medium to suppress the extraction of κ-carrageenan, and to reduce the viscosity of the solution. A relatively high pH was employed in an attempt to maximize the solubility of the protein and minimize its binding to the carrageenans.

The specific activity of all preparations was low and this may be due to difficulty in solubilizing membrane or organelle-bound proteins. The absence of detectable activity in the undialyzed crude extract is probably due to the presence of phosphate, sulfate, and other inhibitory ions. The data of Table IV show that much of the activity was associated with the particulate fraction. Ribi Cell fractionation solubilized about 84% of this activity. The 3-fold increase in specific activity after this step would be consistent with the disruption of organelles or membranes to release the sulfohydrolase rather than increased cell breakage. The present data do not establish the localization of the enzyme within the cell, but in brown algae, biosynthesis of sulfated polysaccharides occurs in Golgi bodies (2). The sulfation of the carrageenan precursor in the red alga, Eucheuma nudum, however, appears to take place in the inner cell wall (5). If the situation in Chondrus is similar to Eucheuma, then the sulfohydrolase would also be in the cell wall. A number of simple compounds were tested as activators with negative results although several inhibitors were noted (Table II). The specific activity observed for the sulfohydrolase, however, compares favorably with that for enzymes involved in the synthesis of biopolymers such as polygalacturonic acid (15), cellulose (1), and peptidyl hydroxyproline (13). Attempts to purify the enzyme further on DEAE-cellulose were not successful due to its instability after the polysaccharide had been separated from the protein.

Inhibition of the sulfohydrolase by λ-carrageenan explains the absence of activity in the crude extracts of diploid C. crispus when tested with μ-carrageenan, and the low activity with ERF as the substrate (Table V). The inhibition further shows that the location of the sulfate ester is important for activity. λ-Carrageenan possesses D-galactose with labile sulfate ester which on alkaline treatment gives 3,6-anhydrogalactose as does μ-carrageenan. Thus, inhibition of the sulfohydrolase suggests either that sulfation on C-4 of the 3-linked unit is required for activity, or that sulfation on C-2 of the 4-linked unit is deleterious. The latter alternative seems less likely as a sulfohydrolase is probably involved in forming ι-carrageenan from υ-carrageenan, and both have sulfate esterified on C-2 of the 4-linked unit.

Different preparations of μ-carrageenan resulted in different levels of enzyme activity with maximum activity occurring when ERF from κ-carrageenan was the substrate (Fig. 2). Because the original κ-carrageenan was isolated with care and there is little likelihood of contamination by μ-carrageenan itself, our enzyme

evidence adds support to the conclusion of Weigl and Yaphe (16), based on chemical conversion, that the ERF represents oligomeric segments of metabolically unfinished κ-carrageenan. The higher sulfohydrolase activity obtained with ERF as compared with μ-carrageenan is a reflection of the lower viscosity, smaller molecular size, and higher molar concentration of the ERF.

Our study confirms the report of Lawson and Rees (6) that G. stellata extracts show sulfohydrolase activity. The higher specific activity of this preparation as compared with that of C. crispus was unexpected. This result may be interpreted in terms of a more active enzyme, or a more suitable substrate. The latter view is favored by the fact that the homogenate of G. stellata is less viscous and has a higher 3,6-anhydrogalactose than that from haploid C. crispus, and thus the endogenous polysaccharide substrate is more like the ERF of κ-carrageenan from the latter source.

A further significant finding was the demonstration of sulfohydrolase activity in diploid C. crispus. It was established earlier that the diploid plants produce neither κ- nor μ-carrageenans (8). We conclude that although diploid C. crispus contains the necessary sulfohydrolase, κ-carrageenan biosynthesis cannot occur because of the absence of a substrate. λ-Carrageenan, the principal constituent of diploid C. crispus, is not a substrate for the sulfohydrolase and therefore remains devoid of 3,6-anhydrogalactose.

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